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(54) Title: GLUCOSE OXIDASE IMMUNOHISTOCHEMICAL DETECTION OF ANTINUCLEAR ANTIBODIES

(57) Abstract

For use in the immunohistochemical detection of antigens and related antibodies in a test serum, an antigen source fixed on a support and stabilized by dehydration treatment with an organic solvent mixture, such as aceton methanol, or mixtures thereof. The support is stored under nitrogen in a sealed polyethylene bag. Further, an immunoenzymatic method for the detection of biological components for a test sample, the method comprising the steps of: contacting the stabilized antigen source with the test sample, contacting the antigen source with an antibody conjugated with glucose oxidase and reactive with the component; incubating the antigen source with a solution containing glucose and a chromagenic mixture; and analyzing the antigen source for the presence of color, preferably with a light microscope.

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GLUCOSE OXIDASE IMMUNOHISTOCHEMICAL DETECTION OF ANTINUCLEAR ANTIBODIES

BACKGROUND OF THE INVENTION

This invention relates generally to immunoenzymatic processes for use in medical diagnoses, and more particularly, to an immunohistochemical process for the detection of antinuclear antibodies in test serum.

In recent years, an increasing amount of research has focused on improving clinical laboratory tests. A primary thrust of this research has been to replace the traditional radiotracer labels with safer and more stable labels.

The research efforts have been quite successful for a number of clinical assays. For example, in tests for antinuclear antibodies (ANA), fluorescent markers have found wide acceptance. While fluorescent markers give quick results, they have not proven entirely satisfactory for a number of assays because once placed in a detectable state they have a very limited shelf life, fading after a few days or more. Moreover, fluorescent assays frequently exhibit high background due to autofluorescence caused by non-specific binding to various proteins, and are impractical for some laboratories because they require the use of relatively expensive fluorescent microscopes.

Another procedure for replacing radiotracer based assays, utilizing enzyme markers in immunoassay and immunohistochemical tests, has been practiced by a number of clinical laboratories in recent years. One of the most commonly used enzymes is horseradish peroxidase, which has been successfully used as a marker in immunohistochemical procedures for the detection of a number of tissue antigens and related antibodies. Due to the relatively small size and high cellular penetration of this enzyme, it has found particular utility in the field of electron microscopy. For the light microscope, horseradish peroxidase has not proven entirely satisfactory, however, because of a high tendency to produce non-specific background stain, even in spite of attempts to quench the presence of endogenous peroxidase-like activity. Another objectionable feature of the peroxidase label is that it generally requires the use of a highly carcinogenic material, diaminobenzidine, for superior stain development. Although substitutes for this material have been developed, their contrast and intensity are typically not entirely satisfactory.

From the foregoing, it will be appreciated that there exists a definite need for a simple, inexpensive, safe and reliable antinuclear antibody detection method that can produce a relatively permanent record of the assay substantially in the absence of non-specific background The present invention fulfills this need.

SUMMARY OF THE INVENTION

The present invention provides an immunohistochemical method which substantially reduces the amount of background stain caused by endogenous materials, while producing a relatively permanent record of the assay.

Moreover, the reagents of the present invention are relatively inexpensive to manufacture, are stable for long periods of time even when stored at room temperature, and have minimal carcinogenic characteristics.

In accordance with the invention, an immunoenzymatic process for the detection of a component of a test sample is provided, said process comprising steps of:

contacting the test sample with an antigen source fixed onto support and stabilized by dehydration;

contacting the antigen source with an antibody conjugated with glucose oxidase and reactive with the component;

incubating the antigen source with the solution containing glucose and a chromogenic mixture and;

analyzing the antigen source for the presence of color from the incubation.

The chromogenic mixtures preferably contain t-nitroblue tetrazolium chloride and m-phenazine methosulfate, which permit analysis with a light microscope. When the component of the test sample is an antinuclear antibody, the antigen source fixed on a glass slide is preferably Hep-2 cells or rat kidney tissue, and the conjugated antibody is goat immunoglobulin reactive with human immunoglobulins.

More particularly, an immunohistochemical method for detecting antinuclear antibodies in a test serum, substantially in the absence of background staining, is provided. This method comprises steps of:

providing a nuclear antigen source fixed onto a slide and dehydrated with acetone at about 4 °C.;

contacting the nuclear antigen source with the test serum;

contacting the nuclear antigen source with goat immunoglobulin conjugated with glucose oxidase and reactive with the antinuclear antibodies;

incubating the nuclear antigen source with a solution containing beta-D-glucose, t-nitroblue tetrazolium, and 1-methoxyphenazine methosulfate;

and analyzing the nuclear antigen source under a light microscope for the presence of a formazan stain.

Another aspect of the invention is a method of stabilizing an immunochemical substrate fixed on a solid support, the method comprising the steps of:

providing a substrate fixed on the support;

contacting the substrate with an organic solvent reagent to substantially dehydrate the substrate;

placing the support into a protective sheath;

backfilling the sheath with a gas; and

sealing the support in the sheath.

The support is preferably a glass slide, and the substrate Hep-2 cells or rat kidney tissue. The organic solvent reagent can be composed of acetone, methanol, or mixtures thereof, and the interacting step performed between about 4 and 8°C. The sheath can be composed of a polyester film, and can contain a dessicant. The preferred gas is nitrogen gas.

Other aspects and advantages of the present invention will become apparent from the following description of the preferred embodiment, which disclose, by way of example, the principles of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Exemplary starting materials useful in practicing the present invention are slides fixed with human epethelial cells (Hep-2) or with rat kidney tissue suitable for use in antinuclear antibody assays. Such slides can be purchased commercially from a number of sources, including Antibodies Incorporated, Davis, California, and Behring, La Jolla, California, or, alternatively, can be manufactured according to conventional techniques. Generally, these slides should be stored at -20°C. to prevent degradation.

In accordance with the present invention, the slides are immersed for about 10 minutes in a bath containing histological grade acetone, methanol, or a combination of these organic solvents. The bath is maintained at a temperature preferably ranging from about 4°C. to about 8°C. After removing the slides from the bath, they are allowed to air dry for about 2 to 3 minutes. Although the dehydration caused by treatment with the organic solvent mixture has significantly stabilized the substrate fixed on the slides, the slides are preferably stored at about 5°C. during continued processing.

After dehydration, the slides are placed into a protective sheath, preferably made from a polyethylene film, such as MYLAR bags with an aluminum outer layer. A small packet containing a standard dessicant is placed in the bag, and the bag is partially sealed. Nitrogen gas is then introduced through the unsealed portion of the bag until the bag bulges slightly. The bag is then totally sealed to prevent the nitrogen gas from escaping.

Goat immunoglobulin conjugated with the glucose oxidase enzyme can be prepared as follows. Glucose oxidase, which is available from a variety of commercial sources, including Sigma, St. Louis, Missouri, and Boehringer Manneheim, Indianapolis, Indiana, is dissolved in distilled water at a concentration of about 12 mg/ml. To this solution is added about 0.2 ml of 0.1 M sodium periodate. The mixture is stirred for about 20 minutes and then dialyzed, preferably against 1 mM sodium acetate buffer, pH 4.4, for about 16 hours. After dialysis, the glucose oxidase solution is mixed with 1 ml of 0.01 M sodium bicarbonate buffer, pH 9.5, containing about 8 mg of the IgG fraction of goat anti-human immunoglobulins (IgG, IgA, and IgM). After stirring for about two hours, 0.1 mls of an aqueous solution containing about 4 mg/ml sodium borohydrate water solution is added. When that reaction has completed (about 2 hours at 4 °C), the entire mixture can be chromatographed on a gel filtration column, such as a Sephacryl S-300, available from Pharmacia, Piscataway, New Jersey, and the fractions containing the enzyme-IgG conjugate pooled. This pool can then be titrated to determine working dilutions.

In accordance with another aspect of the invention, a method for detecting antinuclear antibodies utilizing these reagents is performed as follows. A small amount of test serum, or other test solution, is contacted with the nuclear antigen fixed source on the slide. After about thirty minutes at room temperature, the serum is washed from the slide with phosphate buffered saline (PBS), and then soaked in a cold PBS solution for about 10 minutes. The slides are removed from the PBS and excess moisture blotted with absorbent paper.

A small amount of the enzyme-IgG conjugate, preferably about 50 ul, is contacted with the antigen source for about 30 minutes at room temperature. The slide is again washed with PBS, soaked for 10 minutes in a cold PBS solution, removed, and excess moisture blotted with absorbent paper.

At this time, the color reagent may be prepared. It consists of about 2 volumes beta-D-glucose, 15 mg/ml in 0.1 M sodium phosphate buffer, pH 6.9, one volume t-nitroblue tatrazolium (t-NBT), 2 mg/ml in the same buffer (if necessary, this may be filtered through Whattman No. 1 filter paper just prior to use), and 1 volume of 1-methoxyphenazine methosulfate (m-PMS), 0.8 mg/ml in the same buffer. Although the preferred chromogenic reagents are t-NBT and m-PMS, other materials can also be utilized. To ensure mutarotation of the glucose molecules to their beta form, the mixture may be prepared about 1 hour before the staining reaction.

A small volume, preferably about 100 ul, of the color reagents are contacted wtih the antigen source. The slides are placed in a slide chamber and incubated for about 30 minutes at 55°C. to permit formation of the formazan stain. The excess color reagent is then washed from the slide with PBS, soaked for about 15 minutes in cold PBS and the excess moisture again removed with the absorbent paper.

The slide is then prepared for light microsoope viewing according to standard techniques. Briefly, a glycerol-gelatin mounting medium is placed in a 55°C. incubator for about 5 to 10 minutes for liquefication, and a large drop placed over the antigen source. The antigen source is then covered with a cover slip, taking care to avoid formation of air bubbles.

When the slides are examined under a light microscope, the existence of antinuclear antibodies in the test serum will cause slightly enlarged, homogeneous dark nuclei to appear, substantially without dark background staining.

From the foregoing description, it should be apparent that the present invention provides an effective, inexpensive, highly visible, and permanent record of an antinuclear antibody assay. Further, the nuclear antigen source fixed onto the slide is extremely stable and does not require storage at low temperatures.

While a particular form of the invention has been described in detail with reference to its presently preferred embodiment, it will be understood by one of ordinary skill in the art that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, it is not intended that the invention be limited except by the appended claims.

I CLAIM:

1. An immunoenzymatic process for the detection of a component of a test sample; said process comprising the steps of:

contacting said test sample with an antigen source fixed onto a support and stabilized by dehydration;

contacting the antigen source with an antibody conjugated with glucose oxidase and reactive with said component;

incubating the antigen source with a solution containing glucose and a chromogenic mixture; and

analyzing the antigen source for the presence of color from the incubation.

2. The method of Claim 1, wherein the chromagenic mixture consists essentially of t-nitroblue tetrazolium chloride and m-phenazine methosulfate.

3. The method of Claim 1, wherein said antigen source is Hep-2 cells or rat kidney tissue.

4. The method of Claim 1, wherein said support is a glass slide.

5. The method of Claim 1, wherein said conjugated antibody is goat immunoglobulin reactive with human immunoglobulins.

6. The method of Claim 1, wherein said component is an antinuclear antibody.

7. The method of Claim 1, wherein said analysis is accomplished with a light microscope.

8. A method of stabilizing an immunochemical substrate fixed on a solid support, said method comprising the steps of:

providing a substrate fixed on said support;

interacting said substrate with an organic solvent reagent to substantially dehydrate said substrate;

placing said support into a protective sheath;

backfilling said sheath with a gas; and

sealing said support in said sheath.

9. The method of Claim 8, wherein said support is a glass slide.

10. The method of Claim 8, wherein said substrate consists essentially of Hep-2 cells.

11. The method of Claim 8, wherein said organic solvent reagent is acetone, methanol or mixtures thereof.

12. The method of Claim 8, wherein said contacting step is performed at about between 4 and 8° C.

13. The method of Claim 8, wherein said sheath is a polyester film.

14. The method of Claim 8, wherein said sheath contains a dessicant.

15. The method of Claim 8, wherein said gas is nitrogen gas.

16. An immunohistochemical method for detecting antinuclear antibodies in a test serum substantially in the absence of background staining, said method comprising the steps of:

providing a nuclear antigen source fixed onto a slide and dehydrated with acetone at about 4° C.;

contacting the nuclear antigen source with said test serum;

contacting the nuclear antigen source with goat immunoglobulin conjugated with glucose oxidase and reactive with said antinuclear antibodies;

incubating said nuclear antigen source with a solution containing beta-D-glucose, t-nitroblue tetrazolium, and 1-methoxyphenazine methosulfate; and

analyzing said nuclear antigen source under a light microscope for the presence of a formazan stain.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US82/01107

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL⁴ C12Q 1/68; G01N 33/54

U.S. CL. 435/6,7

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/4,6,7,26,188,260,810;424/3;436/508;3S01,534; 434/296,297

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS, VOL. 66-95, "IMMUNOCHEMISTRY": "HISTOCHEMISTRY": "ANTIBODY", "ANTINUCLEAR".

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	N, AM. J. CLIN. PATH., VOL. 71, ISSUED MAY 1979, S.C. SUFFIN ET AL, "IMPROVEMENT OF THE GLUCOSE OXIDASE IMMUNOENZYME TECHNIC".	2,16
A	N, J.IMMUNOL. METH., VOL 39, ISSUED 1980, J.GUESDON ET AL, "LECTIN IMMUNO TESTS: QUANTITATION AND TITRATION OF ANTIGENS AND ANTIBODIES USING LECTIN-ANTIBODY CONJUGATES".	1-16
A	N,J.HISTOCHEM. CYTOCHEM., VOL. 17, NO. 9, ISSUED 1969, T.E. MASON ET AL, "AN IMMUNOGLOBULIN-ENZYME BRIDGE METHOD FOR LOCALIZING TISSUE ANTIGENS".	1-16
A	N, CLIN. CHIM. ACTA, VOL. 101, ISSUED 1979, NAKAMURA ET AL, "USE OF 1-METHOXY-5-METHYLPHENAZINIUM METHYL SULFATE (1-METHOXY PMS) IS THE ASSAY OF SOME ENZYMES OF DIAGNOSTIC IMPORTANCE".	2,16
X	U.S., A, 3,791,932, PUBLISHED 1974 FEBRUARY 12, SCHUURS ET AL.	1
X	U.S., A, 3,876,504, PUBLISHED 1975 APRIL 08, KOFFLER.	1,4

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
30 NOVEMBER 1982	16 DEC 1982
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	THOMAS G. WISEMAN

Form PCT/ISA/210 (second sheet) (October 1981)

A	U.S., A, 3,997,657, PUBLISHED 1976 DECEMBER 14, DZIOBKOWSKI ET AL.	1-16
A	U.S., A, 3,979,509, PUBLISHED 1976 SEPTEMBER 07, GIAEVER, J	1-16
A	U.S., A, 3,146,163, PUBLISHED 1964 AUGUST 25, BREWER.	8,9,13,14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.